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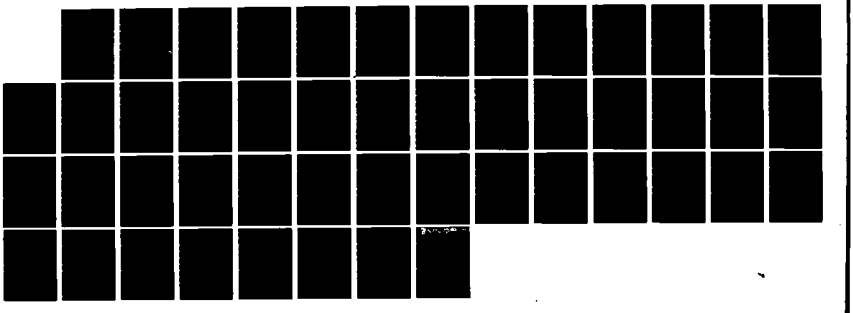

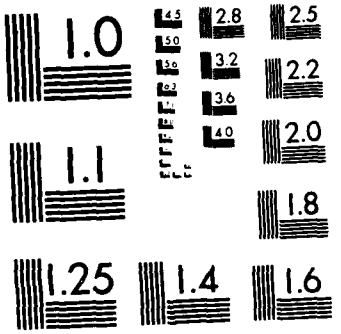

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report reviews progress made under contract DAMD17-79-C-9071. Studies investigated spectrin extractability as method of measurement of RBC viability; use of CPDA-1 to enhance RBC shelf-life and improve "hard-packed" red cell concentrates; and effect of blood collection in CPD, CPDA-1, and CPDA-3 on platelet storage.

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For the protection of human subjects the investigator has adhered to policies of applicable Federal Law 45CFR46.

BLOOD PRESERVATION STUDY

ANNUAL REPORT

E. BEUTLER

January 1980

Supported by

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FORT DETRICK, FREDERICK, MARYLAND 21701

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1. INTRODUCTION

1.1 General Background

The standard red cell preservatives, citrate-phosphate-dextrose (CPD) and acid-citrate-dextrose (ACD) permit red cells to be stored for up to 21 days. This period can be prolonged to approximately 35 days by the incorporation of adenine into the storage medium. Adenine has recently been licensed for use in the United States, as a result of studies sponsored, in part, by the U.S. Army. CPD and ACD result in rapid loss of the organic phosphate ester, 2,3-diphosphoglyceric acid (2,3-DPG) from the erythrocytes. Addition of adenine, while increasing the length of time that red cells may be stored without losing viability, does not improve the stability of 2,3-DPG. Indeed, it slightly accelerates the loss of this compound (1).

Maintenance of 2,3-DPG is of potential importance in the storage of red cells since it exerts a profound effect upon their oxygen dissociation curve (2,3). Red cells that are depleted of 2,3-DPG have an increased oxygen affinity. When they are transfused, their ability to deliver oxygen is somewhat impaired, i.e., a lower tissue oxygen tension is required to extract oxygen from them. However, 2,3-DPG is relatively rapidly replenished after cells are reinfused, one-half normal levels being achieved within about 4 hours, and regeneration being virtually complete at the end of 24 hours (4,5). Although it seems reasonable to suppose that 2,3-DPG is of physiologic importance, it has been very difficult to demonstrate that transfusion of 2,3-DPG depleted cells produce a deleterious effect. Recent investigations provide suggestive evidence that red cells with elevated 2,3-DPG levels

improve cardiac function in baboons in shock, when compared with 2,3-DPG depleted cells (6,7). Furthermore, studies in man suggest that red cells with normal 2,3-DPG may support cardiac output following cardiac bypass surgery in a manner which is superior to that of 2,3-DPG depleted cells (8). While the results of these studies are by no means compelling, it would seem desirable to infuse blood with normal 2,3-DPG content (9).

The use of "frozen blood" has received a great deal of attention in recent years. Claims have been made that red cells stored at sub-freezing temperatures are a superior product, conferring lower risk of hepatitis and of leukocyte sensitization. It must be emphasized, however, as has been pointed out by a National Research Council Committee (8,9), that the superiority of sub-zero stored red cells in these respects remains to be demonstrated. Indeed, the transmission of hepatitis by frozen red cells has been demonstrated (10). With the technology now available, and in the foreseeable future, frozen storage of red cells is prohibitively expensive, and, as pointed out below, frozen storage is quite unsuitable for use under military field conditions.

An additional problem which requires consideration is that of microaggregates. A considerable literature is extant regarding the possible deleterious effect of these particles, which form rapidly when blood is stored in the liquid form (11-16). Microaggregates are believed to be comprised largely of platelets, leukocytes and particles of fibrin, and they may acutely increase the pulmonary artery pressure as they lodge in the lung (17-19). A number of filters have been introduced to remove such microaggregates from stored blood, but some of these

have been shown to be ineffective, and all are quite expensive (20,21). Moreover, these filters markedly limit the rate at which blood can be administered.

1.2 Military significance.

The decrease in mortality among wounded soldiers during recent wars has, in large measure, been due to the large-scale utilization of blood and blood products in the severely injured. The heavy and complex equipment required for the freezing and subsequent deglycerolizing of frozen cells makes frozen storage quite unsuitable for military field purposes, although it may play a role at major military medical installations.

Because of the unpredictable fluctuations in utilization of blood in the military situation, outdating of liquid stored blood is necessarily an even greater problem in the military than in civilian practice. Since large needs for blood might arise at almost any time, sizeable inventories of blood must be kept at hand, and with a 3 week dating period, much of this blood will be wasted. With a 35 day or 42 day dating period, wastage of blood would be less, and logistic problems attendant to collection and delivery of blood would be diminished.

If, in point of fact, 2,3-DPG levels are important in transfusions, they would be particularly so under military circumstances. Here, extensive wounding may result in replacement of the red cell mass several times within a few hours. These critically ill patients are the ones in whom a relatively small change in tissue oxygen tension might make the difference between death and recovery. Microaggregates are believed to be of greatest hazard when large volumes of blood are transfused

to individuals who already have impaired cardiopulmonary function. Wounded soldiers are precisely in this category (13,22,23), and blood which is free of microaggregates might be of valuable improvement over current practice.

1.3 General research strategy of this laboratory.

In view of these considerations, we have developed a 2-pronged strategy of attempting to improve liquid preservation of erythrocytes. Our short-term aim has been to help to provide the data required for early implementation of CPD-adenine solution so that patients may begin to share the benefits of the developments which have already been made. At the same time, recognizing that improving 2,3-DPG preservation and decreasing numbers of microaggregates is an important goal, we are pushing forward with the development of more advanced preservative systems which may combine the ability to maintain viability of red cells for 5 to 6 weeks with these additional advantages.

In carrying out these investigations, increasing consideration has been given to the trend toward fractionation of blood into various formed elements and plasma components. Thus, we have paid considerable attention to the problem of packed erythrocyte storage, as contrasted with the storage of whole blood. One of the most critical needs in the investigation of red cell storage is the development of an in vitro system which has predictive value with respect to the viability of reinfused stored erythrocytes. It is our intention to study a number of in vitro parameters of red cell structure and function at the same time that viability studies are conducted.

2. PROGRESS TO DATE AND CURRENT STATUS OF ONGOING INVESTIGATIONS.

2.1 Study of blood stored in CPD, CPD-A1, CPD-A2, and CPD-A3

2.1.1 Background

CPD (citrate-phosphate-dextrose) has been routinely used as the standard medium for preservation of blood for several years. Red cells from blood which has been collected in CPD can only be stored for 21 days. After this, they are no longer sufficiently viable for transfusion. It has been clearly established that the addition of adenine to CPD preservative solution prolongs the length of time that red cells may be stored to 35 or 42 days (24). Moreover, higher concentrations of glucose than those present in CPD are required to sustain red cell concentrates for prolonged periods of time in adenine-containing media (25).

A preservative solution, originally named CPD-II but now designated CPD-A1 contains 1.25 times the glucose content of CPD and sufficient adenine to provide a concentration of 0.25 mM in the blood-preservative mixture. Higher glucose concentrations were not incorporated into this preservative because of the existence of anecdotal reports that high glucose concentrations increased the rate of fall of the pH of platelet concentrates. CPD-A1 (CPD-II) was studied by a cooperative group (26) with the demonstration that red cells from whole blood stored for 35 days had viability of 80.53 $\pm$ 6.44% ($\bar{x} \pm 1$ S.D.) while red cell concentrates with a hematocrit averaging 75% had a mean viability of 71.38 $\pm$ 10.3% ($\bar{x} \pm 1$ S.D.). After these studies were concluded, it was discovered that the manufacturer of the blood bags had inadvertently prepared the bags used from an obsolete plastic film, PL 130, rather

than the new plastic formulation, PL 146, which had been intended. These studies have now been repeated in PL 146, with results quite similar to those obtained in PL 130. In both their series of studies, it was observed that most or all of the glucose was exhausted from cells in some units packed at hematocrits of 75 $\pm$ 5% and stored for 35 days. There is reason for concern that under field conditions, where the storage temperature may sometimes rise to higher than 4° and where packing of the red cell, a parameter which is difficult to control, may frequently exceed 80%, that CPD-A1 may prove to be suboptimal for the storage of red blood cells. For this reason, two new preservatives, believed to represent improved formulations for storage of red cell concentrates have been devised and manufactured by Fenwal Laboratories. These formulations, designated CPD-A2 and CPD-A3 contain respectively 1.75 and 2.00 times the amount of glucose present in CPD and sufficient adenine to provide a concentration of 0.5 mM in the blood-preservative mixture.

The advantage of CPD-adenine formulations which has been most emphasized has been its effect on the length of time that whole blood or red cell concentrates can be stored without outdating. In some areas, however, outdating is not an important consideration because of geographic factors and because of the nature of the inventory control practices which are employed. The question has often arisen whether such blood banking systems should abandon the use of CPD and implement that of CPD-A1. A second putative advantage of CPD-A1 is that whole blood of red cell concentrates administered near the end of the three week dating period of CPD will be of better quality if CPD-A1 is used as a preservative. The actual existence of this advantage remains to be demonstrated.

Data which have been accumulated over the past 15 years clearly indicate that ATP levels are a disappointing parameter with respect to predicting the viability of stored red cells (27-30). We have also been attempting to identify measurements which may prove to be more useful than ATP in predicting whether or not a given storage system provides red cells which will be viable on reinfusion (31,32).

2.1.2 Studies performed (Jan. 1979 through Dec. 1979)

2.1.2.1 Viability of high hematocrit red cell concentrates prepared from CPD-A1 and CPD blood.

Blood was collected from normal donors into CPD or CPD-A1 and sufficient plasma removed after centrifugation to provide red cell concentrates with hematocrits ranging from 74 to 96%. Glucose, ATP, pH, and 2,3-DPG estimations were carried out using standard techniques developed in this laboratory (33). After 21 days of storage, the red cells were labeled with ^{51}Cr and reinfused into donors in order to determine the viability of the red cells. The results of these investigations are summarized in "The Storage of Hard-Packed Red Blood Cells in Citrate-Phosphate-Dextrose (CPD) and CPD-Adenine (CPDA-1)" by Ernest Beutler and Carol West, BLOOD 54:280-284, 1979. It is apparent that, contrary to some earlier reports (34,35), "hard packed" red cells do not have adequate viability after 21 days of storage, particularly when the hematocrit of the concentrate is over 85%. CPD-A1 provides improved red cell viability, particularly when the hematocrit of the concentrate is very high.

Studies were also initiated comparing packed red cells stored for 28 days in CPD-A1 and CPD-A2. Only one donor was studied with each preservative, and the studies were terminated when a change in

the dating period of the CPD-A2 required Fenwal to withdraw the preservative from circulation. The studies which were carried out are summarized in Table 1. It is apparent that when red cells were packed to a hematocrit of nearly 92% even CPD-A1, containing 1.25 times the glucose content of CPD was insufficient to support the metabolic needs of the erythrocytes. CPD A2, however, containing 1.75 times the amount of glucose present in CPD provided adequate sugar for the storage period. In the course of our investigations of packed cells stored in these media, we had the opportunity of comparing the percentage of high molecular weight cross-linked membrane proteins with the viability of the stored cells. The results of our preliminary investigations in this area are presented in Figure 1. They imply an excellent correlation between the quantity of such proteins and the viability, but the methodology used in these early studies was not entirely satisfactory, and further studies detailed under 3.1 have been initiated.

2.1.2.2 Glycolysis in platelet concentrates prepared from CPD, CPD-A1 and CPD-A3.

In order to determine whether increased glucose concentration adversely affected the storageability of platelet concentrates by hastening the fall of pH, platelet concentrates were prepared in CPD, CPD-A1 and CPD-A3 in order to provide a maximium range of glucose and adenine concentrations for study. The rate of glucose consumption, lactate formation and the net accumulation of hydrogen ions was compared not only to the platelet count of the preservative solutions but also to the residual white cells which were present. The results of these studies are contained in "Platelet glycolysis in platelet storage IV.

The effect of supplemental glucose and adenine" by E. Beutler and W. Kuhl, Transfusion, 20:101-104, 1980. These investigations show that CPD-A3 does not have an adverse metabolic effect on platelets. Moreover, it emphasizes the important role that residual white cells may play in the storage of platelet concentrates. It is likely that the earlier results suggesting that high glucose concentrations might adversely affect platelets may have been artifactual due to increased residual numbers of leukocytes.

2.2 Studies with BAGPM

2.2.1 Background

BAGPM is a bicarbonate-containing preservative medium designed in this laboratory (36,37) for the purpose of maintaining viability and 2,3-DPG levels of packed red cells in a superior fashion. Presumably because of the removal of platelets, granulocytes and fibrinogen, the system minimizes formation of microaggregates (36). The excellent 2,3-DPG preservation observed depends upon buffering of the stored cells with bicarbonate with loss of CO_2 from the system. This can be accomplished either by using containers made from a very permeable plastic film or by introducing into the bags an efficient CO_2 absorbing system. Our investigations suggest that calcium hydroxide, either in small sialastic bags ("baggies") or embedded in sialastic blocks ("blockies"), might be suitable internal CO_2 absorbing devices.

In earlier studies it was demonstrated that $Ca(OH)_2$ added to blood produced marked hemolysis. Hence, leakage of calcium hydroxide from the sialastic membrane "baggie" constituted a serious potential hazard. Therefore, the use of calcium hydroxide embedded sialastic blockies of 3 x 3 x 1 cm size were studied as replacements of the old baggies.

Investigations with varying amounts of calcium hydroxide showed that 6 g calcium hydroxide was the most satisfactory amount to provide adequate preservation of 2,3-DPG without compromising the levels of ATP. It was observed initially that calcium hydroxide shed from the raw surfaces when stored at room temperature in ACD and to some extent also in CPD. These difficulties were accentuated after autoclaving in ACD. They were overcome by providing a coating of liquid sialastic over the surface of the "blockie" and then enclosing it in another sialastic membrane.

All of our developmental studies with BAGPM were performed with blood collected in ACD. In planning the implementation of this system, it became apparent that it would be better to substitute CPD for ACD in the primary container, since platelets collected in CPD can be stored in a more satisfactory manner than platelets stored in ACD. But the final pH of the BAGPM red cell mixture is a critical consideration, and obviously the pH of red cells collected in CPD solution and mixed with BAGPM would be higher than those collected in ACD. Thus, re-investigation of the BAGPM formula was essential.

Recently, Högman and his colleagues have introduced a solution for the storage of red cells, designated SAG (i.e. saline-adenine-glucose) (38). The use of this solution would seem to us to be a retrogressive step (39) because it does little more than to provide additional glucose to the cells and to reduce the viscosity of the cell suspension. BAGPM, in contrast, provides excellent maintenance of 2,3-DPG levels.

2.2.2 Studies Performed (Jan 1979 through Dec 1979)

Blood has been collected in CPD solution and after removal of plasma from the centrifuged red cells 200 ml of BAGPM was added,

using BAGPM solutions in which the bicarbonate/carbonate ratio had been altered without changing the total sodium content. Storage of these samples in the usual BAGPM system with a silcone rubber block containing 6 g of calcium hydroxide was carried out. The results of our initial studies, summarized in Table 2, indicated that ATP preservation was much less satisfactory in all of these units than in those which we had studied previously when red cells collected in ACD were employed. We initially regarded this as being merely a pH effect. However, it was found that now even when the red cells were collected in ACD, ATP preservation was no longer as satisfactory as we had found previously (Table 3). Initially, it was believed that an error had been made by Fenwal in the preparation of the medium or blocks but another possibility also suggested itself. At the time these investigations were initiated, we had begun to store bags in the standing position to conform more closely with practice at most blood banks. In previous studies, the bags had been stored in lying position. As shown in Tables 4 and 5, further studies proved this factor to be essential in adequate ATP preservation. Reverting to storage of red cells in the lying position, we found that satisfactory ATP and 2,3-DPG preservation could be achieved with the original BAGPM formulation (Table 5). Such a preservative is now being prepared by Fenwal.

An investigational new drug application (IND) has been drafted for submission to the FDA for the institution of clinical trials with BAGPM. As pointed out in last year's proposal, certain manufacturing problems had to be overcome, resulting in some delay in the filing of the application and implementation of studies.

2.3 Phosphoglycolate phosphatase in the regulation of red cell 2,3-DPG levels.

2.3.1 Background.

2,3-DPG is formed in red cells from 1,3-DPG through the diphosphoglycerate-mutase reaction. It is broken down through the diphosphoglycerate phosphatase (DPGP) reaction. Clearly, the level of 2,3-DPG in red cells depends upon the balance between these two reactions. One of the unresolved problems, in the understanding of 2,3-DPG metabolism in the erythrocyte has been the fact that the DPGP reaction is a very sluggish one, and it is difficult to account for the rate of 2,3-DPG turnover on the basis of the activity of this enzyme. However, the activity of DPGP is stimulated by many different anions, the most potent of which is phosphoglycolate (39). Recently, it has been demonstrated that small amounts of phosphoglycolate exist in human erythrocytes (40). From this point of view, it is of particular importance that an enzyme which hydrolyzes phosphoglycolate, long known to exist in plants, was recently identified in human red cells (41). Since this enzyme hydrolyzes the activator of the enzyme which degrades 2,3-DPG, its activity might be of great importance in furthering our understanding of the metabolism of 2,3-DPG in health, disease, and in blood storage.

2.3.2 Studies performed (January 1979 through December 1979)

In order to efficiently and accurately assay phosphoglycolate phosphatase activity, we devised a method for the synthesis of ^{32}P labeled phosphoglycolic acid. This technique depended upon incubation of $^{32}PO_4$ in a system containing enzymes and substrates which phosphorylated ADP to radioactive ATP, and then transferred the phosphate to glycolic acid in the backwards pyruvate kinase

reaction. The radioactive substrate was isolated chromatographically, and proved to be both pure and stable.

The activity of phosphoglycolate phosphatase in human red cells was confirmed by incubating hemolysates with the labeled substrates, complexing the inorganic phosphate with molybdate, and then extracting the molybdate into an organic solvent and counting its radioactivity.

With this new assay procedure, it was possible to determine the normal level of phosphoglycolate phosphatase in human erythrocytes and to determine the effect of red cell age on the activity of the enzyme. Examination of the red cells of various species, both mammalian and avian suggested that the interspecies variation of 2,3-DPG levels may be accounted for in part by the phosphoglycolate phosphatase activity of the red cells. These studies are detailed in "An improved assay and some properties of phosphoglycolate phosphatase," Anal. Biochem. 106:163-168, 1980. On the other hand, there was no significant correlation between 2,3-DPG levels and phosphoglycolate phosphatase activity of human red cells and this finding suggested that in man, phosphoglycolate phosphatase activity did not play a limiting role in the regulation of 2,3-DPG levels.

2.4 Spectrin Extractability as a predictor of viability of stored red cells.

2.4.1. Background

A recent study by Lux et al (42) suggested that the extractability of spectrin from red cell membranes might prove to be a sensitive measurement of the arrangement of the spectrin-actin network on the interior surface of the red cell membrane. These investigators found that red cells which had been depleted by prolonged incubation in the absence of glucose had membranes from which spectrin could not be extracted by low ionic strength solutions.

2.4.2 Studies performed (January 1979 through December 1979)

Spectrin extractability from red cell membranes of stored red cells was investigated both by the method of prolonged dialysis of membranes against 1 mM EDTA, as originally described by Lux et al (42) and by a more rapid extraction method consisting of incubation at 37° with hypotonic buffers. The results of these investigations are presented in detail in "Spectrin extractability in blood storage," E. Beutler and D. Villacorte, Transfusion, 21:96-99, 1981. We were unable to confirm the claim by Lux et al that most of the spectrin in severely metabolically depleted red cell stroma could not be extracted by dialysis against low ionic strength solutions. Moreover, even red cells which had been stored for prolonged periods of time in CPD solution, so that they would surely no longer be viable if reinfused, showed entirely normal spectrin extractability.

We conclude that spectrin extractability is not a sensitive means of measuring metabolic damage to red cells, and would be of no value as a predictor of viability in experimental storage systems.

2.5 Publications

The following publications supported, in part, by this contract, appeared during 1979:

Beutler, E.: Red Cell Suspensions. N Engl J Med. 300:984 (Letter), 1979.

Beutler, E., West, C.: The Storage of "Hard-Packed" Red Blood Cells in Citrate-Phosphate-Dextrose (CPD) and CPD-Adenine (CPDA-1). Blood 54:280-284, 1979.

Tegos, C., Beutler, E.: Platelet Glycolysis in Platelet Storage: I. The Glycolytic Enzymes. Transfusion 19:203-205, 1979.

Beutler, E., Kuhl, W., Tegos, C.: Platelet Glycolysis in Platelet Storage: II. Levels and Turnover of Metabolic Intermediates. Transfusion 19:467-471, 1979.

Tegos, C., Beutler, E.: Platelet Glycolysis in Platelet Storage: III. The Inability of Platelets to Utilize Exogenous Citrate. Transfusion 19:601-603, 1979.

3. Proposed Studies

3.1 Viability studies of human red cells stored in CPD-A1 and CPD-A2.

We plan to investigate the length of time that whole blood and red cell concentrates packed to a hematocrit of 75 $\pm$ 5 can be stored after collection in CPD A-2. These studies will be performed jointly with Lieutenant Colonel Carl Peck and Lieutenant Colonel Robert Bolin of the Letterman Army Institute of Research (LAIR) using a protocol very similar to the one which was used for the investigation of CPD-A1 (see Appendix 1). First, these investigators will study the effect of storage for 35 days. If the average viability of blood and packed cells stored for 35 days exceeds 72%, studies will be carried out after 42 days storage. If viability at 42 days is satisfactory, studies will be carried out also at 49 days storage. After these studies have been completed at LAIR, we will undertake to confirm, in 12-15 normal subjects, the limit of storageability both for whole blood and for red cell concentrates at hematocrit 75$\pm$5%.

The relationship between hematocrit and storageability of packed red cells from blood collected in CPD-A1 and CPD-A2 will be compared. The media are already covered by the IND of Fenwal Laboratories and approved by our institutional committee. Sequential studies will be carried out of whole blood and red cells packed to 90% hematocrit and stored in CPD-A1 and CPD-A2. The methods of study to be used will be identical to those described by Beutler and West, BLOOD, 54:280-284, 1979. Blood collected from 5 normal donors will be studied after 28 days storage in CPD-A1 and from 5 other donors in CPD A-2. If viability of these stored cells exceeds an average of 72%, investigations will also be carried out after 35 days storage,

using 5 donors for each preservative medium.

3.2 Investigations of Red Cells Stored in BAGPM

Once an IND has been obtained, the viability of red cells stored in BAGPM will be investigated in normal volunteers. 450 ml of whole blood will be drawn into 63 ml of CPD solution. After centrifuging at 1000 g for 9 min at room temperature, all of the visible platelet rich plasma will be expressed and will be replaced with BAGPM in the closed, sterile system provided by Fenwal. This solution will consist of 190 ml of water containing 4.84 mM sodium carbonate, 130.53 mM sodium bicarbonate, 1.05 mM adenine, and 29 mM mannitol. Ten ml of 1.1 M glucose in 20 mM Na $H_2PO_4 \cdot H_2O$ will be added to the blood from a plastic sac integral to the system. The red cell suspension in BAGPM will be stored in a lying position at 4°. It will be mixed once weekly. At the end of the storage period of 35, 42, or 49 days, 20 ml of the mixed red cell suspension will be removed from the bag with sterile precautions and placed in a 50 ml serile plastic tube. Chromium ^{51}, 0.6 microcuries per kilogram body weight, will be added to the mixture and it will be allowed to stand at room temperature for 20 minutes. Sterile physiologic saline will be added to make a total volume of 50 ml. The suspension will be centrifuged, the supernatant removed, and the packed red cells washed again in 40 ml of sterile physiologic saline solution. The packed, washed red cells will then be resuspended in an equal volume of physiologic saline and infused into the normal volunteer subject. Samples will be drawn at 0, 5, 10, 15, and 20 min and 24 h and the viability computed. A complete IND will be submitted before these studies are initiated.

3.3 Further in vitro studies in BAGPM

Fenwal laboratories is now studying a new plastic film designated V-776 which is more permeable to gas than the polyvinyl chloride films which had heretofore been used. Since the pH-stabilizing effect of BAGPM depends upon loss of CO_2 from the storage suspension, it is recognized that a suitably permeable bag may obviate the necessity for a CO_2-absorbing block in the red cell suspension. Blood from 5 donors will be collected in CPD, placed into bags composed of V-776. After centrifugation at 1000 g for 9 min, all visible supernatant plasma will be pressed off and 200 ml of BAGPM, as described under 3.2 will be added. The red cell suspension will be mixed weekly and estimations of red cell 2,3-DPG, ATP, and pH (4°) will be made over a 42 day period.

3.4 Investigation of the relationship between high molecular weight cross-linked proteins and viability.

The studies detailed under 3.1 and 3.2 will provide us with an opportunity to study the heretofore uninvestigated parameter of red cell storage viz. the measurement of high molecular weight crosslinked stromal proteins. Such cross-linked proteins have been reported to occur in patients with certain types of hemolytic disorders, and may produce inflexibility of the membrane (42,43). Hemoglobin-free membranes will be prepared by hypotonic lysis and washing the membranes in 10 mM tris buffer, pH 7.4. The membrane preparations will be dissolved in a solution containing1% SDS and 2 mM EDTA in 50 mM tris buffer, pH 7.4. Electrophoresis will be carried out in a 2.6% polyacrylamide 0.3% agarose gel containing 1% SDS in a tris-EDTA-acetate buffer, pH 7.4 and being 11 cm in length. After electrophoresis for 2-2.5 hours

at 5 milliamperes, the gels will be stained with Coomassie blue. An aliquot of the membrane lysate will also be treated with 100 mM β-mercaptoethanol and electrophoresed in gels containing 10 mM β-mercaptoethanol. The difference between the amount of high molecular weight material obtained with and without β-mercaptoethanol will represent the high molecular complex bound together by disulfide bridges. The material which is present after β-mercaptoethanol treatment presumably represents material held together by cross-linking peptide bridges.

As shown in last year's report, studies with the ektacytometer fail to show the development of rigidity of red cells during storage. A more sophisticated ektacytometer is being constructed by Technicon under direction of Dr. Marcel Bessis, and this year, we expect to receive a prototype instrument which will be used for further studies.

3.5 Studies of Platelet Preservation

Further in vitro studies of the preservation of platelets will be undertaken. The investigations detailed by Beutler and Kuhl, Transfusion, 20:101-104, 1980 indicated that a major portion of the fall of pH during platelet storage could be attributed to contaminating leukocytes. We propose to investigate the effect of performing a second centrifugation to remove contaminating white cells on the storageability of platelet concentrates.

Preliminary experiments have shown that a centrifugation of 500 g for 10 min, and the platelet rich plasma pressed over into a satellite bag. The satellite bag will be centrifuged at 500 g for 5 min and the supernatant plasma containing most of the platelets will be transferred into another satellite bag. A platelet concentrate will be prepared from this second platelet rich plasma by centrifugation at 3000 g for

20 min, removing all but the 60 ml of the plasma and then resuspending the platelet pellet.

At each stage of this procedure, the yield of platelets and the number of contaminating leukocytes will be determined by standard counting methods. The platelet suspensions will then be stored on a platelet agitator at room temperature. Daily samples will be studied to determine the rate of fall of pH, the accumulation of lactate and pyruvate, and the shape of the platelets will be evaluated under phase microscopy. The rate of these changes will be compared with the changes which occur in platelet concentrates prepared from platelet rich plasma which was not subjected to a second centrifugation to remove leukocytes. Approximately ten units of platelets stored with and ten without the additional centrifugation will be investigated. A decreased rate of lactate formation, fall of pH, and development of spheroidicity of platelets would point toward the possibility of improved platelet preservation times.

4.0 BUDGET

4.1 Personnel	% of Time	Salary	Benefits	Total
E. Beutler, M.D.	25	$18,000	$4,500	$22,500
Mrs. C. West	100	20,000	2,200	22,200
Dishwasher	50	4,300	473	4,773
Total				49,473

4.2 Supplies		
^{51}Chromium	1,000	
Biochemical Reagents	4,000	6,000
Glassware	1,000	
4.3 Donor Fees for in vivo and in vitro studies		4,000
4.4 Laboratory Fees and physicians fee for physical Exam (CBC, SMA-12, etc.)		2,000
4.5 Publications, instrument maintenance		4,000
4.6 Indirect Costs *		26,092
4.7 Total		91,565

*40.0% of modified total direct costs. Negotiated with DHEW 9/21/79.

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FIGURE 1

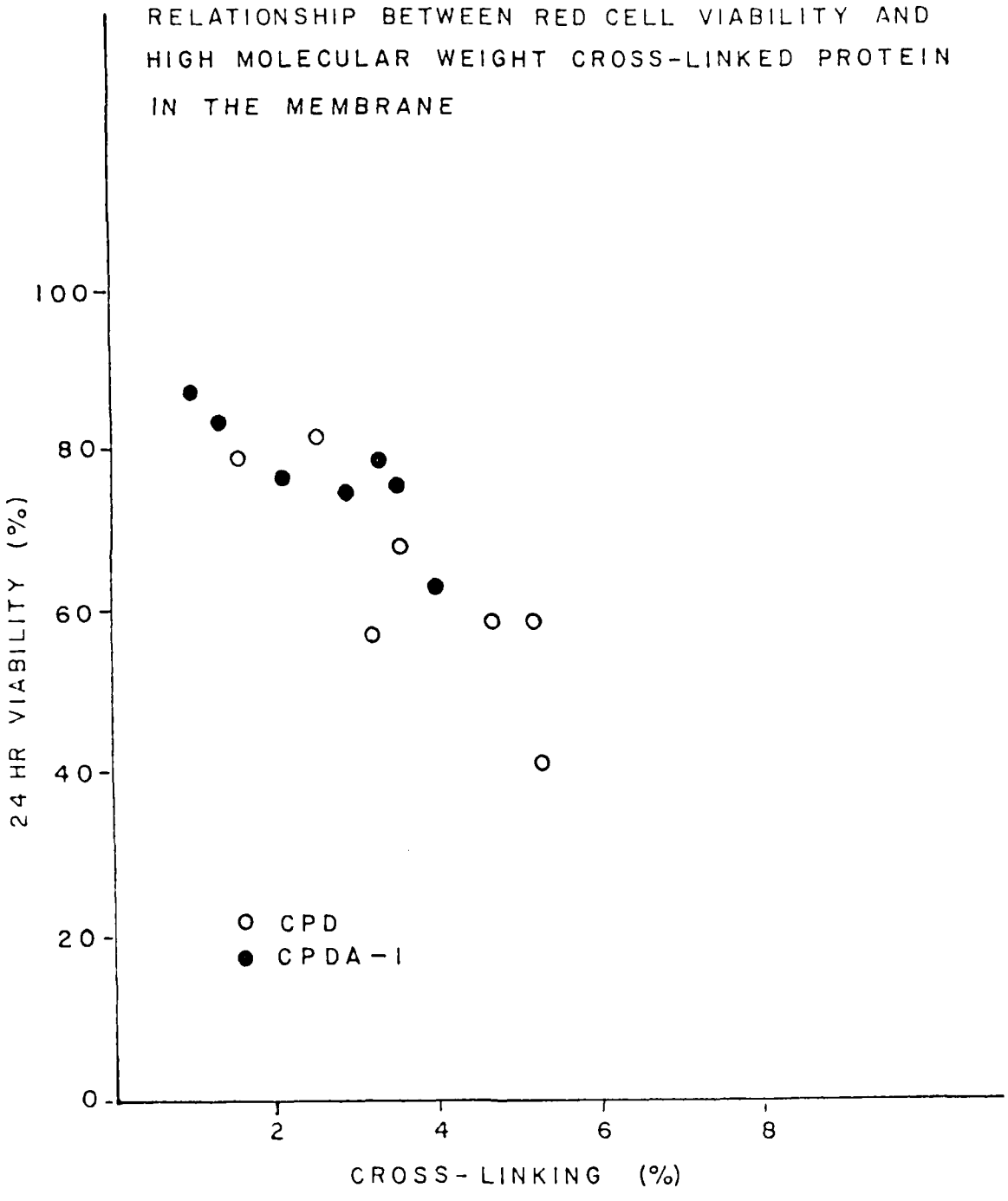
RELATIONSHIP BETWEEN RED CELL VIABILITY AND
HIGH MOLECULAR WEIGHT CROSS-LINKED PROTEIN
IN THE MEMBRANE
24 HR VIABILITY (%)
100
80
60
40
20
0
2
4
6
8
CROSS-LINKING (%)
CPD
CPDA-1

TABLE 1

Comparison of the Storage of "Hard-Packed" Red Cells from Blood Collected In CPD-A1 and CPD-A2

Packed Cells Stored 28 Days		CPD-A1 CR	CPD-A2 CL
HCT (%)	Initial	91.8	93.3
	Final	91.5	92.5
pH (4°C)	Initial	7.47	7.48
	Final	7.02	7.08
Glucose (mg%)	Initial	465.8	572.6
	Final	0.1	134.3
ATP (µMoles/ gm Hb)	Initial	4.33	5.51
	Final	2.39	4.47
GSH (µMoles/ gm Hb)	Initial	7.91	7.49
	Final	6.88	6.86
Viability (24 hrs, %)	Initial	-	-
	Final	58.8	85.2

TABLE 2

The Storage of Red Cells in BAGPM from Blood Collected in CPD

Medium in Primary Bag	CPD			CPD			CPD		
Mixing Schedule (x/week)	Unmixed			1x/week			5x/week		
$NaHCO_3$ in BAGPM (mM)	130.53			130.53			130.53		
Na_2CO_3 in BAGPM (mM)	4.84			4.84			4.84		
Storage Position	Standing			Standing			Standing		
Donor	K.N.			F.Z.			C.A.		
Day of Storage	ATP	pH	2,3 DPG	ATP	pH	2,3 DPG	ATP	pH	2,3 DPG
0	4.91	7.89	15.71	4.32	7.89	14.22	5.35	7.90	15.74
7				0.93	7.92	21.30	0.77	8.03	19.37
14				0.97	7.85	26.92	0.64	7.99	17.97
21				1.10	7.60	13.98	0.90	7.67	14.26
28				0.79	7.59	12.65	0.50	7.74	12.29
35	1.70	7.42	5.71	0.95	7.54	15.41	0.70	7.71	13.93

ATP and 2,3 DPG values are given in µMoles/g Hb.

TABLE 3

The Storage of Red Cells in BAGPM from Blood Collected in CPD and ACD

Medium in Primary Bag	ACD	CPD	CPD
Mixing Schedule (x/week)	1x/week	1x/week	1x/week
$NaHCO_3$ in BAGPM (mM)	130.53	113.68	113.68
Na_2CO_3 in BAGPM (mM)	4.84	13.26	13.26
Storage Position	Standing	Standing	Standing

Donor	B.L.			D.O.			J.M.		
Day of Storage	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG
0	4.40	7.47	15.86	3.72	7.77	16.55	4.18	7.79	17.1
7	2.23	7.62	16.95	0.93	7.65	15.65	0.80	7.77	15.6
14	1.91	7.56	20.20	0.95	7.64	16.09	0.65	7.81	16.2
21	1.62	7.63	17.12	1.1<	7.68	17.26	0.59	7.54	-
28	1.65	7.61	16.10	1.01	7.50	17.62	0.65	7.63	-
35	1.42	7.75	20.98	1.11	7.56	18.84	0.43	7.71	-
42	1.34	7.74	20.60	1.19	7.59	-	0.35	7.76	-

ATP and 2,3 DPG values are given in µMoles/g Hb.

TABLE 3

(Continued)

Medium in Primary Bag	CPD			CPD			CPD		
Mixing Schedule (x1/week)	1			1			1		
$NaHCO_3$ in BAGPM (mM)	122.1			130.53			140.2		
Na_2CO_3 In BAGPM (mM)	8.95			4.84			0		
Storage Position	Standing			Standing			Standing		
Donor	J.S.			S.L.			B.T.		
Day of Storage	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG
0	3.96	7.66	16.11	3.72	7.59	16.44	3.54	7.53	20.6
7	1.05	7.64	20.41	0.97	7.72	15.04	1.19	7.62	20.1
14	0.90	7.76	14.3	0.62	7.80	16.07	0.86	7.74	19.9
21	0.97	7.69	17.3	0.52	7.83	17.69	0.73	7.79	20.9
28	0.89	7.66	17.5	0.40	7.80	16.13	0.53	7.89	20.6
35	0.69	7.79	23.8	0.42	7.99	16.61	0.55	7.89	18.3
42	0.76	7.77	23.4	0.33	8.05	15.79	0.45	7.98	14.

ATP and 2,3 DPG values are given in µMoles/g Hb.

TABLE 4

Effect of Storage Position on Red Cells Stored in BAGPM from Blood Collected in CPD

Medium in Primary Bag	CPD			CPD			CPD		
Mixing Schedule (x/week)	1			1			1		
$NaHCO_3$ in BAGPM (mM)	13<.53			130.53			130.53		
Na_2CO_3 in BAGPM (mM)	4.84			4.84			4.84		
Storage Position	Lying			Lying			Lying		
Donor	E.L.			E.P.			P.G.		
Day of Storage	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG
0	4.26	7.82	11.2	3.83	7.87	12.7	4.84	7.85	11.6
7	1.66	7.83	14.6	1.30	7.86	16.3	1.69	7.85	11.6
14	1.76	7.76	13.1	1.43	7.78	14.2	1.79	7.76	15.1
21	2.06	7.71	14.5	1.54	7.71	16.5	1.93	7.71	16.4
28	1.90	7.70	14.2	1.52	7.69	10.6	2.17	7.67	11.9
35	2.10	7.67	13.2	1.95	7.66	12.7	2.28	7.62	9.6
42	2.60	7.62	10.9	2.46	7.59	7.7	2.80	7.54	8.6
49	2.90	7.59	9.5	2.49	7.53	7.3	2.80	7.49	6.5

ATP and 2,3-DPG values are given in µMoles/g Hb.

TABLE 4

(Cont.)

Medium in Primary Bag	CPD			CPD			CPD		
Mixing Schedule (x/week)	1			1			1		
$NaHCO_3$ in BAGPM (mM)	130.53			130.53			130.53		
Na_2CO_3 in BAGPM (mM)	4.84			4.84			0		
Storage Position	Lying			Lying			Lying		
% of Usual Volume BAGPM	30			60			60		
Donor		A.Y.			M.P.			H.C.	
Day of Storage	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG
0	4.27	7.92	14.4	4.33	7.99	18.21	5.63	7.973	-
7	1.92	7.82	16.3	2.50	7.84	21.0	2.02	7.910	-
14	2.00	7.70	16.9	2.71	7.69	20.7	2.05	7.750	-
21	2.03	7.60	17.9	3.13	7.57	15.4		7.54	-
28	2.13	7.65	17.0	3.26	7.51	10.9	-	-	-
35	2.26	7.48	14.7	3.13	7.27	7.1	-	-	-
42	1.72	7.48	-	2.89	7.29	-	-	-	-

ATP and 2,3-DPG values are given in μMoles/g Hb.

TABLE 4

(Cont.)

Medium in Primary Bag	CPD			CPD			CPD		
Mixing Schedule(x/week)	1			1			1		
$NaHCO_3$ in BAGPM (mM)	130.53			130.53			130.53		
Na_2CO_3 in BAGPM (mM)	4.84			4.84			4.84		
Storage Position	Standing			Standing			Lying		
Donor	G.S.			E.Y.			R.M.		
Day of Storage	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG
0	4.04	8.15	-	3.18	7.99	20.3	3.59	7.92	15.8
7	0.93	-	-	0.96	7.93	22.4	1.70	7.84	23.2
14	1.02	7.95	-	1.09	7.92	19.4	1.85	7.77	21.9
21	0.96	7.84	-	1.06	7.83	20.9	1.86	7.73	23.5
28	0.99	7.78	-	1.32	-	20.0	1.86	-	19.9
35	1.04	7.74	-	0.58	7.73	16.7	1.66	7.63	19.7
42	0.99	7.69	-	-	-	-	1.75	7.66	-

ATP and 2,3-DPG values are given in μMoles/g Hb.

TABLE 4

(Cont)

Medium in Primary Bag	CPD
Mixing Schedule (x/week)	1
$NaHCO_3$ in BAGPM (mM)	130.53
Na_2CO_3 in BAGPM (mM)	4.84
Storage Position	Lying

Donor F.Z.

Day of Storage	ATP	pH	2,3-DPG
0	4.15	8.13	-
7	0.83	-	-
14	0.89	7.87	-
21	1.04	7.83	-
28	0.97	7.71	-
35	1.12	7.63	-
42	1.05	7.57	-

ATP and 2,3-DPG values are given in μMoles/g Hb.

TABLE 5

Storage of Red Cells in BAGPM from Blood Collected in CPD in Lying Position

Medium in Primary Bag	CPD			CPD			CPD		
Mixing Schedule (x/week)	1			1			1		
$NaHCO_3$ in BAGPM (mM)	124			124			130.53		
Na_2CO_3 in BAGPM (mM)	8			8			4.84		
Storage Position	Lying			Lying			Lying		
Donor	J.L.			F.V.			C.S.		
Day of Storage	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG	ATP	pH	2,3-DPG
0	3.87	8.10	11.4	4.44	7.98	12.8	5.63	7.97	15.4
7	1.49	7.92	11.9	2.03	7.81	15.1	2.15	7.75	16.2
14	1.70	7.77	12.1	2.09	7.72	15.5	2.34	7.64	17.7
21	2.02	7.63	9.6	2.06	7.67	16.8	2.64	7.60	18.9
28	2.33	7.54	6.1	2.50	7.62	13.9	2.77	7.50	14.8
35	2.27	7.46	5.3	2.69	7.59	13.3	2.50	7.40	14.5
42	2.22	7.33	-	2.74	7.42	-	2.13	7.34	-

ATP and 2,3 DPG values are given in µMoles/g Hb.

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APPENDIX 1

I. IDENTIFICATION FOR PROTOCOL NUMBER 9-79

A. TITLE: Human In-Vivo Evaluation of Blood Preserved in CPDA-2.

B. IND: BB-IND 1087 Modified Anticoagulant CPD with Adenine.

C. SPONSOR:
Fenwal Laboratories
Division of Travenol Laboratories, Inc.
One Baxter Parkway
Deerfield, Illinois 60015

D. INVESTIGATORS:
Carl C. Peck, M.D.
Chief, Blood Research Division
Letterman Army Institute of Research
Presidio of San Francisco
San Francisco, California 94129
(415) 561-5875

Ernest Beutler, M.D.
Scripps Clinic and Research Foundation
Chairman, Department of Clinical Research
10666 North Torrey Pines Road
La Jolla, California 92037

E. MONITOR:
D. H. Buchholz, M.D.
Medical Director, Fenwal Laboratories
(312) 948-2733 (office)
(312) 546-6311 ext. 2497 (office)
(312) 526-7582 (home)

II. BACKGROUND

The use of adenine as an additive for blood preservation was proposed in The Federal Register by the Bureau of Biologics on January 20, 1978. The proposed rule described a formula which was tested by Fenwal Laboratories, Division of Travenol Laboratories, Inc. and approved by the Bureau on May 12, 1978. The approval included the storage of Red Blood Cells (Human) and Whole Blood (Human) for 35 days in CPDA-1 solutions. A Final Rule was then published in the Federal Register August 4, 1978 allowing CPDA-1 to be added to the list of Federally approved anticoagulants.

The improvement of blood storageability from 21 days in CPD to 35 days in CPDA-1 is due to the presence of 17.3 mg of adenine plus slightly more dextrose (1.25 times more dextrose than CPD) in 63 ml of anticoagulant solution. The known values for red cell survival studies in CPD at 21 days are approximately 80% while the values for CPDA-1 at 35 days are approximately 78% according to the results of the study that was the basis of approval for CPDA-1 in PL-146 by Fenwal Laboratories. An acceptable survival value has been considered to be greater than 70%.

This protocol describes a study to test the storageability of blood that has been preserved in a new anticoagulant (CPDA-2) consisting of twice

the amount of adenine as is present in CPDA-1 plus 1.75 times the amount of dextrose in CPD. Whole blood and packed red blood cells will be stored at 4°C for 35, 42, 49 and 56 days to determine the maximum storage time of erythrocytes preserved in anticoagulant CPDA-2 solution.

III. SCOPE OF STUDY

Each participating laboratory will study up to 40 normal donors to determine in-vivo survival of autologous red blood cells labelled with ^{51}Chromium following storage for up to 56 days in anticoagulant CPDA-2. Each laboratory will initially test 35 day storage periods with 5 units of whole blood and 5 units of packed red blood cells (hematocrit 75±5%).* If the results show survival values of greater than 70%, 10 additional units (5 whole blood and 5 packed cells) will be tested after 42 day storage. Based upon these studies, additional storage times of 49 and 56 days will be evaluated as indicated.

IV. MATERIALS AND METHODS

A. Blood Containers

Blood Pack Units used in this study will be made of PL-146 plastic and will contain 63 ml of a sterile, non-pyrogenic solution consisting of:

Dextrose (hydrous), USP	2.81	g
Sodium Citrate (hydrous), USP	1.66	g
Citric Acid (hydrous), USP	206	mg
Sodium Biphosphate, USP	140	g
Adenine	34.6	mg

The Blood Pack Units will be a triple bag design with a plasmapheresis adaptor for use in later studies, if necessary for obtaining platelets. The code number will be FX-756.

B. Donor Selection

1. All donors must meet published standards of the Bureau of Biologics. During aspects of clinical trials, the provisions of AR 70-25, Use of Volunteers as Subjects of Research, and Protection of Human Subjects, Federal Register, Volume 39, No. 105, May 30, 1974, will apply and be adhered to strictly by all investigators. No persons will donate or be transfused with any blood component under this protocol unless informed consent has been obtained prior to donation.

   All containers for whole blood or red cells intended for later autologous infusion shall bear a label with identification of the donor/recipient. Prior to reinfusion, the donor/recipient shall verify that he (she) has identified his (her) signature on the label.

*"Hard-packed" units with hematocrits of 90±5% will also be studied.

2. In Vitro Tests of Donors

In addition to tests required to determine whether a candidate meets the donor standards, the following tests will be performed prior to blood collection into the investigational product:

- hematocrit
- hemoglobin
- glucose 6-phosphate dehydrogenase screen
- examination of peripheral smear
- white cell count
- sickle cell hemoglobin screen
- pregnancy test on all females

Significant abnormality of any of the above studies or a positive pregnancy test of females shall exclude a donor.

C. Instructions for Blood Collection: Use Aseptic Technique

1. Identify Blood-Pack Unit using appropriate donor identification system. Confirm that all numbered tubing of each Blood-Pack Unit contains its own identical segment numbers.

2. Adjust donor scale to desired collection.

3. Suspend pack from donor scale as far as possible below donor arm and clamp donor tube with hemostat.

4. Apply blood pressure cuff and disinfect site of phlebotomy.

5. Inflate blood pressure cuff to 60 mm Hg.

6. Remove needle cover and accomplish phlebotomy; release hemostat.

7. MIX BLOOD AND ANTICOAGULANT AT SEVERAL INTERVALS DURING COLLECTION AND IMMEDIATELY AFTER COLLECTION.

8. Collect 450 ml of blood.

9. Apply hemostat to donor tube. Seal donor tube with handsealer clip between hemostat and primary pack. Cut between hemostat and clip; collect pilot samples from donor.

10. Release pressure in cuff and withdraw needle.

11. Strip blood from donor tube into pack, mix and allow tube to refill. Seal at X marks on donor tube to provide numbered aliquots of anti-coagulated blood.

12. Store filled unit between 1° and 6°C.

D. In-Vitro Analysis of Stored Whole Blood and Red Blood Cells

On day 0 and the final day of storage, the following values will be determined on each stored unit:

Plasma glucose	Plasma Sodium
Plasma Hemoglobin	Plasma Potassium
Plasma pH	Plasma Ammonia
Red Cell 2,3-Diphosphoglycerate	Red Cell Adenosine Triphosphate
Red Cell Hematocrit	

Other In-Vitro tests may be performed at the discretion of the investigator.

E. Survival Studies

On the final day of storage an aliquot of autologous red blood cells will be labelled with ^{51}Chromium and returned to the volunteer donor. Appropriate venous samples will be obtained to determine the 24 hour post-transfusion red blood cell survival.

These precautions are essential and apply to both whole blood and red blood cells:

1. Storage of 1°C to 6°C within a 2°C range should be maintained until just before transfusion. Contents of the Blood-Pack units shall not be mixed during storage.

2. The blood should be inspected immediately before use and rejected if there is abnormal color or appearance.

3. Medication should not be added to the blood.

4. Before administration, the container and the intended recipient will be carefully identified.

5. Three to five days prior to the anticipated reinfusion of autologous ^{51}Cr-tagged RBCs, a sample of blood from the blood bag will be subjected to standard aerobic and anerobic bacterial culture. The resultant cultures must be negative and a gram stain of plasma drawn from the bag on the day of reinfusion must not show evidence of bacterial contamination.

If an unexpected severe adverse reaction is encountered by an investigator, the clinical monitor will be notified by telephone as to the nature and severity of the adverse reaction. Notification will be provided by Travenol to the Food and Drug Administration, Bureau of Biologics. Determination of what constitutes a severe adverse reaction will be the responsibility of the principal investigator with direct medical responsibility for the infusion resulting in the reaction.

V. SUBMISSION OF DATA

Each investigator will complete the data sheets provided and upon completion of each donor study will mail the original sheets to:

D. H. Buchholz, M.D.
Medical Director
Fenwal Laboratories LCI-01
One Baxter Parkway
Deerfield, Illinois 60015

Photocopies will be retained by each investigator.

VI. ANALYSIS OF DATA

Survival values of blood stored as either Whole Blood (Human) or Red Blood Cells (Human) for 35, 42, 49, or 56 days will be compared to known acceptable values of > 70% as a measure of an adequate transfusion. Plasma pH, ammonia, sodium, potassium, glucose and hemoglobin will be measured along with erythrocyte adenosine triphosphate and 2,3-diphosphoglycerate and the values compared with those seen following shorter storage periods in CPD or ACD (21 days storage) or CPDA-1 (35 days storage) anticoagulant.

If survival values and In-Vitro data analysis show promising results at any of the storage intervals tested further studies may be undertaken.

VII. DURATION OF STUDY

This study is expected to be completed within one year.

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